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DETERMINING SEX IN POSTHATCHLING LOGGERHEAD SEA TURTLES USING MULTIPLE GONADAL AND ACCESSORY DUCT CHARACTERISTICS

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ABSTRACT: The sex of young sea turtles is difficult to determine because they lack externally dimorphic characteristics and heteromorphic sex chromosomes, yet internal dimorphic morphology is defined at hatching. We tested the reliability of nine internal gonad and accessory duct characteristics to identify the sex of 558 posthatchling loggerhead sea turtles accurately. We modified existing laparoscopic procedures, previously used to classify the sex of larger sea turtles and other turtle species, for use in posthatchlings. Here we describe our approach and quantify the reliability of our morphological criteria. Sex was verified by histological examination of gonadal biopsies from a subset of the turtles. We noted seasonal shifts in early gonadal structure so that some characters which were reliable in the summer and fall were not reliable other times of the year. Thus, we confined the analysis to the six characters that were reliable year round: gonad shape, paramesonephric duct size, gonad size, paramesonephric duct lumen presence, paramesonephric duct mobility, and gonad attachment. Using discriminant analyses of the biopsy and morphological data, we found high correlations between sex from tissue biopsies and these six morphological characters; this analysis misclassified just 2% of the animals with histological verification. Applying the classification functions to animals without histology and comparing those results to our visual classification resulted in 2% reclassification. The analysis reclassified animals that we or the histology correctly identified that had both female-like and male-like characters. Our method provided accurate identification of sex in very young sea turtles. This methodology enables sex ratio assessments in early life stages, which are critical to species recovery efforts. Additionally, sex assignment data are basic to our understanding of patterns and processes directing dimorphic changes of the gonads and their ducts.

Key words: *Caretta caretta*; Cheloniidae; Sea turtle; Sex determination; Sex ratio; Testudines

MARINE turtles, like many turtle species, lack heteromorphic sex chromosomes (Bull, 1980; Ewert, 1991). Sex is determined by the incubation environment (primarily temperature) during the middle third of incubation (Miller, 1997; Valenzuela, 2004; Wibbels, 2003; Yntema and Mrosovsky, 1980). Loggerhead seaturtle (*Caretta caretta*) eggs incubated at constant temperatures produce females at warmer and males at cooler temperatures (Miller, 1997; Mrosovsky, 1988; Yntema and Mrosovsky, 1980). Sex and sex ratios for clutches and even entire nesting sites are often estimated indirectly from nest temperatures, beach temperatures (Godfrey and Mrosovsky, 1999; Godley et al., 2001a), and/or nest incubation durations (Godley et al., 2001b; Marcovaldi et al., 1997) and rainfall (Godfrey et al., 1996), rather than by direct examination of the hatchlings' gonads. In several studies, hatchlings were sacrificed to include gonadal histology (Godfrey et al., 1996; Mrosovsky et al., 1984*a*, 1984*b*; Mrosovsky and Provancha, 1992), but sample sizes, temporal extent of the study, and/or geographic ranges were limited, often because loggerheads are a protected species (IUCN, 2004; U.S. Department of the Interior and U.S. Department of Commerce, 1978) so destructive sampling is restricted.

The sex of juvenile turtles >20 cm Straight Carapace Length (SCL) and adult turtles is frequently determined by hormonal assay, by gross morphology of the gonad during postmortem exam, or in live turtles >30 cm curved carapace length (CCL), by laparoscop-

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ic examination of the gonads, with or without biopsy histology (Miller, 1997; Wibbels et al., 1987; Wibbels et al., 1991). Blood hormone analyses include testosterone titers (Miller, 1997; Owens et al., 1978; Wibbels, 1999; Wibbels et al., 2000) and estrogen-to-testosterone ratios. Radioimmunoassay of serum testosterone levels is restricted to use when ambient water temperatures exceed 23 C; below that temperature this hormone assay is not reliable (Braun-McNeill et al., 2000; Wibbels, 2003; Wibbels et al., 2000). Hormone ratios in chorioallantoic/amniotic fluid of individually isolated eggs also diagnose sex (Gross et al., 1995).

Hormone assay methods were not useful in very small sea turtles (<15 cm SCL) because radioimmunoassay requires larger volumes of blood than could be safely obtained and because hormone concentrations are too low to discriminate sex even in pooled blood samples (Owens, 1999; Owens et al., 1978). Until recently, sexing very small turtles by laparoscopy was precluded because probe sizes were too large, and there were no morphological characters that could distinguish male from female gonads at this stage of development. In this study, we show that small laparoscopic probes can now be used safely. We also identify gonadal and accessory duct morphological characters that reliably diagnose sex in posthatchling sea turtles.

Methods

Animals

Loggerhead hatchlings (n = 1048) were collected as they emerged from 91 natural nests laid on beaches from North Carolina (2002–2003) to Florida (2002–2004). These clutches were deposited throughout the entire nesting season. Hatchlings typically emerge over several successive nights; we collected hatchlings from the first major emergence of each nest and transported them in Styrofoam™ chests to rearing facilities at Florida Atlantic University (FAU), Duke University Marine Laboratory (DUML), and Mote Marine Laboratory (MML) where they were raised to a size (85–88 mm SCL) and mass (120 g) we identified in preliminary studies as sufficient for safe laparoscopy. Turtles at this size have little or no residual yolk, and their gonads and accessory ducts can be found easily. The turtles at each facility were raised for 2.5–6 mo (depending on hatch date and water temperatures) in individual containers in flowing seawater at 24–28 C. The tanks received 12D:12L fluorescent "full spectrum" lighting positioned no more than 45 cm above the water's surface. Turtles were fed daily on chopped shrimp, Mazuri® carnivorous reptile gel, and an in-house manufactured gel diet (modified from Stamper and Whitaker, 1994; Stokes et al., 2006).

Laparoscopic Exam and Gonadal Identification

When turtles reached 120 g, they were fasted for 24–36h and prepared for laparoscopy. The body caudal to the head was washed with water, antimicrobial soap and povidone iodine scrub solution, and then the incision site for the laparoscope's entry through the inguinal fossa was scrubbed with 2–4% chlorhexidine solution. We injected 0.25 ml of 1 mg/ml lidocaine (1.3–2.1 mg/kg) around the incision site in the anterior right inguinal fossa 10–30 min before we made the incision. With the turtle held head down and with the right rear leg extended and adducted, a longitudinal 4–6 mm-long incision was made through the skin with a scalpel. Next, the skin was spread and a stab incision was made with surgical scissors through the inguinal muscle and coelomic membrane. We then conducted the laparoscopic exam (modified from Rostal et al., 1994; Wibbels et al., 1987) using a 2.7 mm diameter, 30° rigid orthopedic endoscope that was inserted through the incision. After identifying the caudal end of the lung, the scope was swept caudally and slightly dorsally to locate and examine the gonads and their laterally positioned ducts. Up to nine internal anatomical characteristics were recorded for the gonads and their accessory ducts (Table 1). We found it unnecessary to insufflate posthatchlings with air. The presence of peritoneal fluid made some mobility and shape characters easier to see.

After the laparoscopy was completed the incision was closed with 1–3 simple interrupted sutures, most often of chromic gut or

TABLE 1.—Variables examined and scored to determine loggerhead turtle sex. The six characters that were reliable for year-round use are designated by ** and are listed in order of importance. The categorical descriptions for each variable: Gonad shape, Paramesonephric duct (PD) size, Gonad size, PD lumen presence, PD mobility, and attachment of the gonad to the coelomic wall.

Character	Descriptive categories
Gonad size **	Small / Large
Gonad color	White / Cream / Yellow
Gonad shape **	Fusiform / Flat and Thin / Loose and Folded / Robust
Gonad surface	Large granules / Small Granules / Both
Vascularization	Low / Moderate / High
Attachment **	One edge / Asymmetric / Midline
Paramesonephric duct size **	Small / Large
Paramesonephric duct mobility **	Mobile / Immobile
Paramesonephric duct lumen **	Present / Absent

Maxon®, 3-0 (Govett et al., 2004). At two rearing sites, we supplemented the closure with surgical glue as recommended by the veterinarian. Triple antibiotic ointment was applied to the site, and then each turtle was coated with a water-based lubricating gel and held out of water overnight. The length of the procedure from the incision to suturing was 5–25 min. Turtles were returned to the water and fed the next day; typically, they ate enthusiastically.

When deemed appropriate by the attending veterinarian, a prophylactic dose of the antibiotic Ceftazidime (20 mg/kg) was administered subcutaneously (SQ) in one shoulder. Butorphanol (0.1 mg/kg SQ), an analgesic, was administered in the other shoulder of half of the animals ~10 min before surgery. Permit conditions allowed the use of Butorphanol only at one rearing site.

In preliminary studies, we held 40 turtles for 2 or 4 wk following laparoscopy with or without biopsy to determine postoperative recovery time. One week was sufficient for incision healing, the resumption of normal behavior, feeding, and growth (Govett et al., 2004; Wyneken, unpublished data). Thus, as weather permitted, all but 12 of the 1048 turtles were released into the Florida or Gulf Stream currents 1–3 wks after surgery. Those from DUML were released between at ~34 N lat, 78 W lon; FAU turtles were released between 24–26 N lat and 77–78 W lon, and MML turtles between 24 N lat and 81 W lon.). The 12 not released were transferred to other facilities for education or further research.

Histological Verification

We use gonadal histology to verify (i) our visual discriminations of sex, (ii) our descriptions of each sex-specific characteristic, and (iii) the statistical reliability of our analysis. We assessed our accuracy in determining sex using visual criteria by an independent histological examination of a subsample of the sexed turtles (one or two individuals per nest, selected randomly). Because of the unknown effects of biopsying very young turtles, permitting restrictions limited the proportion of animals that could be biopsied. Tissue (a 1–2 mm biopsy) was collected from the cranial third of each turtle's right gonad during the laparoscopic exam using laparoscopic biopsy forceps. This site was chosen to minimize potential impact on the future vas deferens or epididymus of the males and on potential germ cell recruitment from the caudal gonad (Limpus, unpublished data). Biopsies were preserved in 10% buffered formalin, prepared as paraffin sections, and stained with hematoxylin and eosin (H&E) for light microscopy.

The stained sections were classified as males and females by standard histological criteria. Turtles were males if the cortex was poorly developed (usually one cell layer thick) lying on a thin fibrous membrane; this is the tunica albuginea reported by Yntema and Mrosovsky (1980) in testis stained with PAS, and when the medulla was formed by tubules. Developing seminiferous tubules, seen in cross section, were bordered by strongly basophilic nuclei organized around the periphery with weakly basophilic cytoplasm

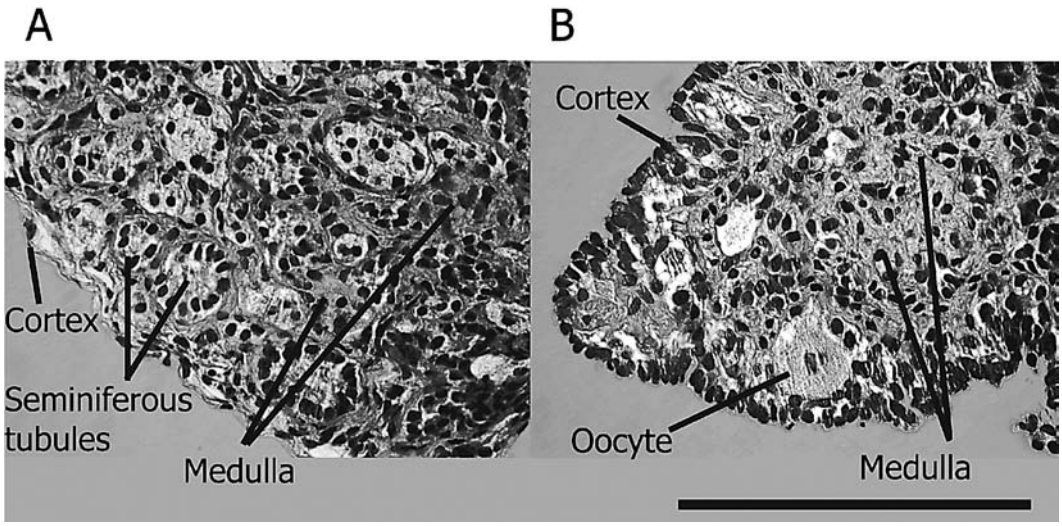

FIG. 1.—Loggerhead immature testis (A) and ovary (B), H&E stained (200×). The testis is characterized by a poorly developed cortex and well organized medulla formed of seminiferous tubules and stroma. Ovaries have a cortex of basophilic cells (primordial germ cells, developing follicles and primary oöcytes); the medulla is poorly organized. Scale bar = 500 μ

toward the center of each (Fig. 1A). Females were identified by a disorganized medulla and the presence of a well developed cortex formed by two to five layers of basophilic cells (Wyneken et al., 2003). There were some larger developing oöcytes, identified by their basophilic nuclei, multiple darker staining nucleoli and lightly staining cytoplasm (Fig. 1B). The presence of a well developed tunica albuginea in the females (Yntema and Mrosovsky, 1980) was not distinct in H&E stained ovaries and hence was not used here.

Sex Specific Criteria

In 2002, we started by scoring just four variables (gonad shape, color, vascularity, and surface characteristics) based on published criteria for turtles and other vertebrates (Parenti and Grier, 2004; Limpus, 1992; Merchant-Larios, 1999; van der Heiden et al., 1985; Wyneken, 2001). However, there were unanticipated seasonal changes in three of these initial variables in the middle of 2002, so we added five additional characters for a total of nine variables (Table 1). Those nine were scored for the remaining 2002 turtles and all of the 2003 and 2004 turtles.

Statistical Analyses

The nine internal anatomical characteristics were coded for discriminant analysis (SAS® ver. 9.1), a multivariate procedure used to determine group membership. Three characteristics changed with season but six were seasonally invariable. From these six, we developed a training data set (biopsied turtles with all six characters scored) and a test data set (turtles without biopsy, but scored for all six characters) for the discriminant analysis. The training data set was used to create classification functions in the discriminant analysis to predict the sex of turtles. These functions then were applied to the test data set to predict the sex of turtles that were not biopsied. Data were not transformed, the covariance matrices formed for the discriminant analysis first were tested for homogeneity of variances, and we used equal priors for the classifications.

We assessed our accuracy of sex assignments in three ways. First, we directly compared our visual assignment of sex with biopsy results from a subset of animals. Next we used the discriminant analysis to rank the factors that formed the best classification functions and determined the accuracy of

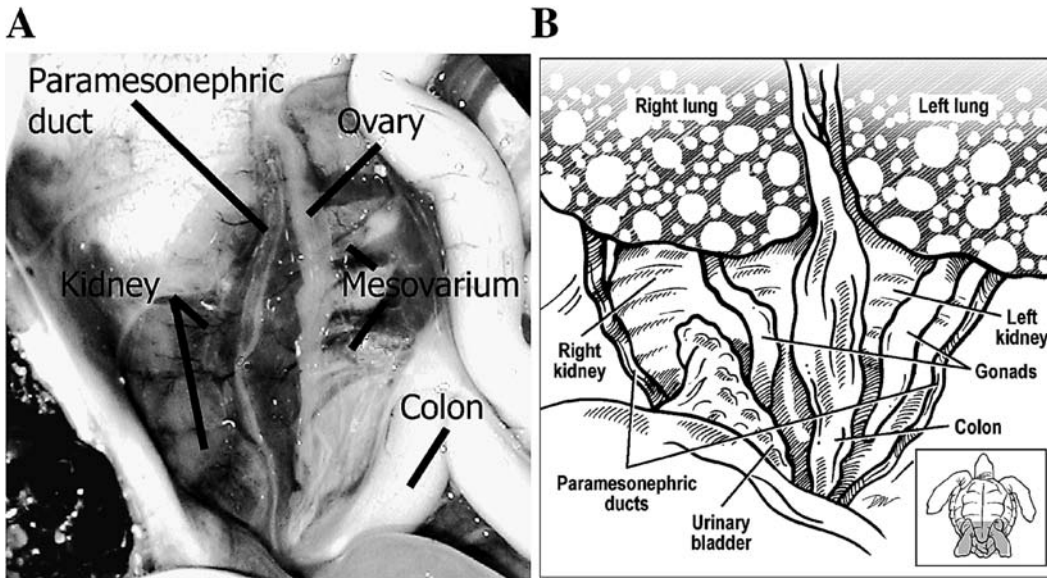

FIG. 2.—A. Loggerhead right immature ovary, overlying the right kidney (cranial is toward the top). A mobile paramesonephric duct with a large lumen lies lateral to the gonad. Gonads and kidneys are just posterior to the lungs which lie deep to the intestines. B. Diagram (posteroventral view) of the gonads, kidneys, and paramesonephric ducts, all located caudal to the lungs (drawn by D. Witherington).

the discriminant analysis by comparing how many turtles in the training data set the analysis misclassified when compared to the biopsy results. Lastly we compared the predicted sex assignments of the test data set with our visual assignments of the same animals.

RESULTS

The paired gonads are located within the coelomic cavity, caudal to the lungs and attached to the peritoneum overlying the kidneys. We were able to distinguish males and females using multiple gonadal and accessory duct characteristics (Fig. 2). Of the 1048 turtles examined, we biopsied 244 (biopsied turtles) and scored all six characters in 137 of those biopsied (scored & biopsied turtles). In the 244 biopsied turtles we visually identified 66 male and 178 female. In the subset that comprised 137 scored & biopsied turtles we found 34 male and 103 female. Those 137 scored & biopsied turtles formed the training data set for the discriminant analysis. The 421 scored turtles were visually identified as 97 male and 324 female; these formed the test data set for the discriminant classification.

Shape

We found that testes tended to be compact and fusiform (in all but three males). They showed little mobility. In contrast, all but four females had ovaries that were supple and folded upon themselves, when observed in peritoneal fluid, and ovaries generally appeared larger in area than testes (Figs. 2–3) when compared in similar sized animals. Gonads observed in the summer and fall were robust or full-bodied while those seen in winter and spring tended to be flimsy leaving them thin and flat against the body wall.

Color

Color varied from yellow (testes) to cream (either ovaries or testes). In the winter months both became white as vascularity decreased (Fig. 3 E–F).

Surface Characteristics

During winter and spring, testes were inactive, not growing, and appeared smooth. In the summer and fall testes were active and the smooth surfaces appeared reticulate (Fig. 3 C, Fig. 4 A) because of the actively differentiating seminiferous tubules. Year-

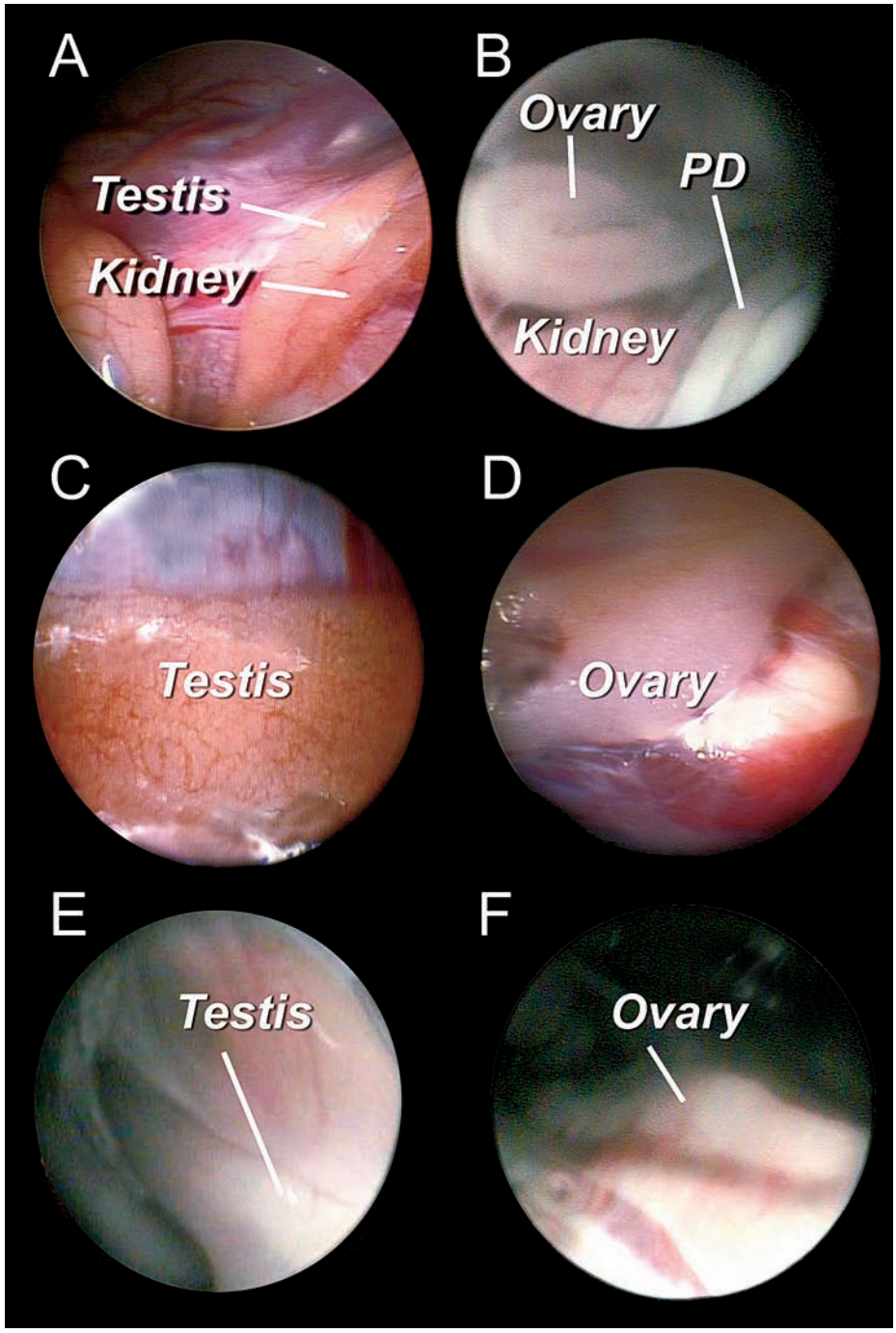

FIG. 3.—Laparoscopic images of (A) a normal fusiform testis attached to the coelomic wall overlying the kidney, (B) an immature right ovary (folded) with a large mobile paramesonephric duct (PD) located laterally to the right. (C) close up of the testis showing reticulated appearance of surface, (D) an ovary showing the smooth surface composed of small cells, (E) The caudal half of a winter testis that is thin, white and almost translucent and (F) Lateral view of a winter ovary; thin and white. These gonads are approximately 1.7 cm in length. Magnifications are approximately A–B: 2×, C: 6×, D: 4×, E–F: 3×.

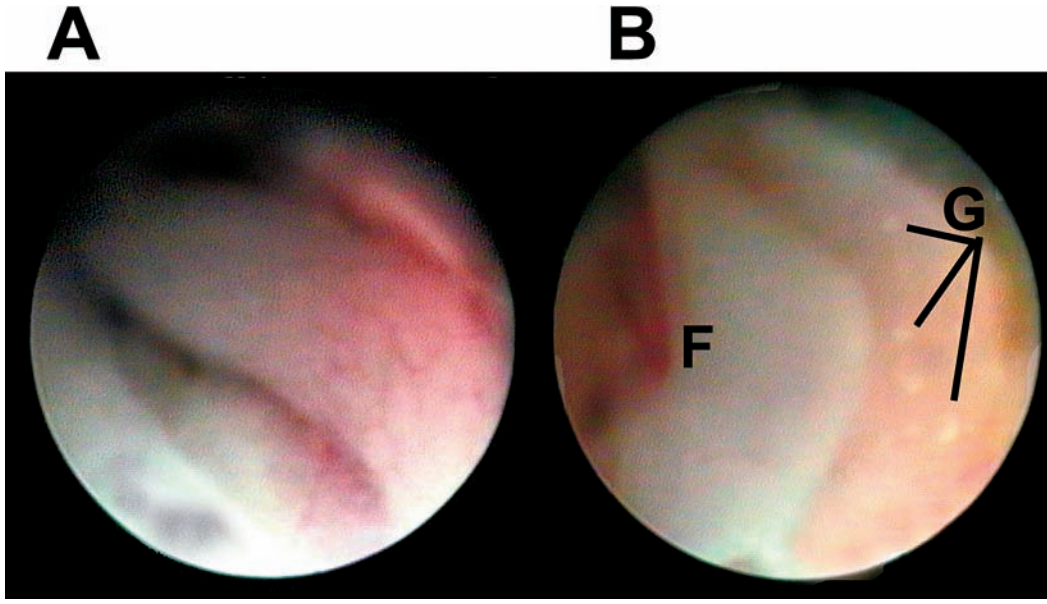

FIG. 4.—Laparoscopic images of (A) A fusiform testis has a reticulated pattern on its surface. This caudal half of the testis also is beginning to lose vascularity with the winter season. (B) An ovary switching from active fall (yellow) to winter (white with large granules) morphology. There are large granules visible on right (G). It is attached asymmetrically along is long axis to the coelomic membrane. The narrower medial side (left in picture) is folded (F) over the lateral portion. Magnifications are approximately A–B: 6×

round, ovaries had smooth surfaces, but the small cortical cells gave the surface a granular appearance (Fig. 3 B, D). In the winter and spring ovaries (and occasionally testes) often had large granules in the surface (Fig. 4), and the number varied greatly from a few to > 40. These were identified histologically as either lymph vessels or developing follicles; grossly the two structures appeared similar.

Attachment

Attachment to the coelomic wall differed between the sexes. Testes were attached tightly along their longitudinal midlines by a very short mesorchium. Attachment of the ovary was more variable. Each ovary was suspended by the mesovarium running longitudinally along one edge or asymmetrically along the long axis of the gonad (Fig. 4B); however, some ovaries were attached along the midline as well.

Paramesonephric Duct Size, Mobility, and Lumen

Lateral to each gonad and kidney was a paramesonephric duct (PD; Fig. 2). The PD size, lumen presence or absence, and mobility were all useful characters. The lumen was incomplete in more than half of the males. Just nine of 154 histologically identified females scored for this character lacked an obvious lumen. Large, mobile PDs (Fig. 2 A) were found in the majority of females, 125 of 139 females, compared to just five of 50 males scored for both characters. The PDs of males tended to be small and immobile.

Vascularization

Vascularization was identified as high when blood vessels were seen in all parts of the gonad, moderate when blood vessels were small and restricted to just the ends of the gonads, or low when the vessels within the gonad and within the mesovarium or mesorchium appeared small and without blood. During the winter and early spring months, gonadal vascularity decreased (Fig. 3 E–F); the gonads became thin and white and often developed discrete large white granules in their surfaces. They ceased to grow while other somatic growth continued. Because of this seasonal shift in vascularity and the

consequential variability associated with the effects of low vascularity, color and surface granularity were not useful characters year round.

Histology

Histological characteristics in cortex structure and medulla organization distinguished testes from ovaries (Fig. 1). While some gross sex-specific characters shifted seasonally, the relative differences between the males and female cortex and the medulla organization persisted year-round so that winter and early spring samples were still accurately identified. In the 244 biopsied turtles, histology showed 65 male and 179 female. Our visual identifications of these biopsied turtles compared favorably with our histological results (97% overall), differing in just seven turtles: five in 2002 and two in 2003. Our accuracy increased over time (percent correct: 86% (2002), 92% (2003), and 100% (2004)).

After exploring the data, we determined that color, vascularity, and granules all change with season and were eliminated from further analysis. Gonad shape, PD size, gonad size, PD lumen presence, PD mobility, and gonad attachment (listed in order of importance) were the discriminating variables. The discriminant analysis of the training data set (histological results of scored & biopsied turtles: 35 male and 102 female), using within-groups covariance matrices (the covariance matrices were significantly different and could not be pooled), was significant ($P >$ Wilks' Lambda <0.0001) and was able to correctly classify all but one male in the 2002 yr class and two females in the 2003 yr class, and no turtles were misclassified in the 2004 yr class. The misclassified male had male-like gonadal features but female-like duct characteristics. The misclassified females had some male-like gonadal and duct characters; attachment was questionable, the PD was small, and the lumen was not obvious. Sex was predicted for 421 scored turtles without biopsies in the test data set and was based on the above classification functions. These results were compared to our visual assignments of sex and differed in just 10 animals (2%). Those 10 all had some male-like and some female–like characteristics. Using a stepwise discriminant analysis, we found that using five of the six characters, omitting only attachment, proved nearly as accurate (97% in the test data set). Omitting shape and gonad size from the six – the two most difficult variables to score, but retaining attachment allowed for 93% accuracy in classification for males and 87% accuracy for females.

Survivorship

Only five turtles died related to the laparoscopic study. Two of these mortalities were difficult to link to the procedure, as the incisions had healed and the turtles were feeding and behaving normally before developing fungal pneumonia while awaiting release. Just prior to laparoscopy, one animal lapsed into a coma and died following injections of Butorphanol and lidocaine. Both of these drugs are processed by the liver. Preexisting liver disease was identified upon postmortem exam. Two other turtles, each with idiopathic adhesions of the intestine to the body wall, died from systemic infection following intestinal perforation and repair; these two turtles were the only ones whose deaths could definitely be attributed to the surgical procedure. Two other turtles experienced puncture of the intestinal tract but recovered after surgical closure of the hole. One of these turtles shared the same idiopathic adhesion as we found in one of the turtles that died; he was from the same clutch.

Two turtles from the FAU colony were transferred to an educational facility for display. They remain alive and well at the time of writing. Ten turtles from the DUML colony were transferred to another research lab where they were in captivity for 18–24 mo prior to their offshore release.

Discussion

There was a learning curve in the first few months of this study that required us to look beyond published key morphological features and identify more characteristics than expected. Seasonal changes in gonad morphology required scoring additional variables towards the middle of the first year of data collection. As a result, the analyses reported here include fewer turtles than were examined during the first year, yet the re-

duced sample size was sufficient to allow us to assess the procedure and determine accuracy.

Once we focused primarily on both the gonads and the paramesonephric ducts, sex identification became highly reliable during visual exams. In females, typically the ovaries are attached asymmetrically or by one edge. The female PD is usually large and mobile with a large lumen. Male PDs are usually small or no obvious duct is present; when the duct is present, it lacks a complete lumen. The discriminant analysis correctly identified all but one male in the training data set; he had large, mobile ducts. The PD tends to regress in males but persists as the Müllerian duct in females where it is destined to transport gametes (Miller and Limpus, 2003). Given their lack of function in males it is not surprising that the PDs are smaller, and each lacks a complete lumen. The PD continues to grow in females so its larger size is consistent with expectations. The maintenance of a lumen is also expected. The mobility of the PDs in these young loggerheads foretells the mobility of the oviducts in mature females.

Our dismissal of color, vascularity and granularity was necessary because there was a major reduction in the perfusion of the gonads with blood during the late fall and winter. When perfusion dropped, the gonads became pale, sometimes translucent. Lymph vessels swelled primarily in females (rarely in males), and some cortical cells continued their growth in females, producing the appearance of large granules (Fig. 4). The gonads also became smaller, but the larger ovaries/smaller testes relationship remained, so gonad size could be retained in the analysis.

Our visual assignment accuracy, verified with histology of the biopsied turtles, rose throughout the study. Even so, overall our visual misidentification rate, when compared to biopsy results, was only 3%. Quantitatively, the discriminant analysis did little better, misclassifying 2% in the training data set (scored & biopsied turtles). The comparison of the classified, 421 scored animals in the test data set with our visual identifications yielded only a 2% difference. These results show that the sex of posthatchling sea turtles can be accurately identified visually.

Previous methods of estimating sex ratios, particularly incubation duration and nest temperature, while of value, none-the-less include limited ground-truthing, limited spatio-temporal coverage, and/or significant levels of hatchling sacrifice (Wibbels, 2003). Dead hatchlings remaining in nests after an emergence may provide sex ratios, but these may not be the same as the sex ratios of surviving hatchlings because incubation temperature is not uniform throughout the nest. The periphery and core may differ significantly (Kaska at al., 1998; Maxwell et al., 1988) and it is unknown from where in the nest dead hatchlings come. Radioimmunoassay of hormone titers, often ground-truthed by laparoscopy (Wibbels, 1999), can be used reliably in loggerhead turtles larger than 20 cm SCL (Dellinger et al., 2004); it is not effective in young loggerhead sea turtles because of their low levels of circulating hormones (Owens, 1999) and not practical because of exsanguination risk. While assay of hormones in chorioallantoic/amniotic fluids appears to be promising in some species, the technique cannot be used with fully in situ nests (Gross et al., 1995) and is ineffective in olive ridley (*Lepidochelys olivacea*) seaturtles (Merchant-Larios, 1999). Our procedure circumvents all of these shortfalls because it does not require hormone assays, animal sacrifice, individual egg tracking, or assumptions about the relationships between incubation characteristics and sex ratio.

There are physical limits to the procedure. These include: (1) turtles must be large enough to accommodate the 2.7 mm laparoscope and (2) there must be enough space between the laparoscope, and the gonad and ducts so that the critical characteristics can be observed. In these young turtles, residual yolk must be absorbed so that it does not block the view of the scope. We studied turtles that were at least 120 g; however dissections of dead turtles as small as 80 g (72–75 mm SCL) suggest that smaller loggerheads could be examined using this method as the internal yolk sac is mostly depleted (2–3 mo of age) and the body is of sufficient size to accommodate the scope. Finally, rearing large numbers of hatchlings for several months and maintaining their health is labor intensive,

expensive, requires adequate space and adequate water supply; hence husbandry may be limiting in some circumstances, as well. However, for studies of restricted coverage, these resource needs would not be so great.

Our protocol makes it possible, for the first time, to accurately identify sex ratios in young sea turtles during the year that they enter the ocean without sacrificing the animals. Such early documentation of sex ratio allows for real-time recognition of sex ratio. Visual identification of the gonads using laparoscopy is a reliable method for identifying sex and monitoring the gonadal changes in large numbers of turtles with little mortality risk (< 5 deaths/1048 turtles). Of the 12 turtles we transferred to other facilities, the two educational display turtles remain alive, well, and are growing 4 yr after their examinations. Of the 10 that were transferred to a different research facility, eight were subjected to second, unrelated, laparoscopic exam without biopsy at one yr; their gonads and ducts were also examined during the procedure. There were no obvious differences in the gonads and ducts of these turtles compared with those that had not been biopsied. These turtles and the two not examined further remained healthy and were released by their facility into the Gulf Stream after 1.5–2 yr in captivity.

We cannot directly address if there are any long term impacts to the reproductive potential of the animals subjected to laparoscopy with or without biopsy because they do not mature for 25–30 yrs. However, breeding success is well documented for older and larger turtles whose gonads were examined laparoscopically. Several species involving more than 100 animals have been laparoscopically examined, with or without biopsy. Many were tracked for months as they underwent migrations, maturation to breeding, and during breeding events (Limpus, 1992; Limpus et al., 2005; Miller et al., 1998).

We expect that sex ratios observed in these turtles brought into the lab should mirror those of their wild cohort, particularly given the relatively short time between hatching and reaching size sufficient for laparoscopic exam. There is no evidence for sex-specific mortality over this short time span.

In our case, the sex ratio data will allow us to update life history models and assess methods of estimating sex ratios based on nest temperatures. Our procedure opens the door for the development and rigorous quantitative testing of two-sex population models such as those being developed for loggerhead management. Additionally, early detection of sex ratio shifts may provide a mechanism for in depth investigation of the sex determination process without large scale sacrifice of young turtles.

This study is the first to rigorously identify gross sexual dimorphic characters of posthatchling turtles. While we used these characters in the context of laparoscopic examination, the morphological characteristics we identified should also be diagnostic of sex under other situation where gross gonadal and duct morphology may be observed such as in post mortem examinations or museum specimen dissections.

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